Evaluation of Bioactivity and Preliminary Phytochemical Investigation of Herbal Plants Against Ampicillin Resistant Bacteria

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Abstract: Presently, natural products are extensively used as substitute of synthetic drugs because of natural and environment friendly antimicrobial actions due to presence of bioactive compounds. These bioactive compounds are found extensively in herbs. In this study, bioactivity and phytochemical study of crude extracts of *Ocimum basilicum, Thymus vulgaris, Rosmarinus officinalis* and *Origanum vulgare* against ampicillin resistant bacterial strains isolated from fruits and vegetables eaten as raw, was investigated. Bacterial strains were isolated from the surface of selected washed fruits and vegetables by serial dilution method. Three morphologically different strains (A, P and L) were selected on the basis of resistance to ampicillin (150ug/ml). 16s rRNA sequencing revealed that bacterial strains A, P and L belong to *Pseudomonas aeruginosa* strain A, *Pseudomonas aeruginosa* strain P and *Rhodobacter sphaeroides*, respectively. Phytochemical screening showed presence of different chemical compounds such as alkaloids, tannins, saponins, phlobotannins, quinones, coumarin and flavonoids. Occurrence of saponins and flavonoids in the extract was further confirmed by thin layer chromatography (TLC). *Ocimum basilicum, Rosmarinus officinalis* and *Origanum vulgare* extracts caused inhibition of the isolated ampicillin resistant organisms (*Pseudomonas aeruginosa*) but effect of *Thymus vulgaris* was more pronounced. Anti-mitotic study revealed the ability of these extracts to reduce dividing cells by the anti-mitotic properties and this is helpful in inhibition of the development of cancer cells. Therefore, these plants can be used to discover natural products that will aid in more effective developments of new drug research activities.

Keywords: Medicinal plants extracts, phytochemical screening, antibacterial activity.

INTRODUCTION

Recently, World Health Organization (WHO) reported that nearly 50 to 80% of world population is depending upon the medicine of plant origin for dealing with different diseases [1]. Antimicrobial compounds of plants have an exceptionally great therapeutic potential. They are compelling in the medication of irresistible ailments while at the same time alleviating a number of the reactions that are frequently connected with synthetic antimicrobial [2]. Utilization of medicinal plants for the cure of various diseases is as old as humanity itself [3]. Indeed, today medicinal plants contribute as a modest source of medications for the world's population.

Herbal medicine, which is often referred to as herbalism or plant prescription, is the utilization of herbs for their helpful or medicinal quality [4]. Herb is a plant or plant part esteemed for its medicinal, sweet-smelling or flavorful qualities. Natural plants have a variety of chemical substances that have effect on the body. Natural drug is the most seasoned type of health awareness known framework to humanity. Almost all countries in the world have an expertise concerned with the therapeutic properties of the local flora [5]. Medicinal floras are well-known due to remedial potentials of certain natural dynamic substances, which are present in them. These compounds or phytochemical constituents incorporate terpenes, flavonoid, bioflavonoid, benzophenones, xanthenes and also a few metabolites, for example, tannins, saponins, cyanates, oxalate and anthrax-quinones [6].

Latest research has demonstrated that sweet basil is a successful cancer inhibition agent [7]. In different studies basil indicated higher cancer prevention agent adequacy as compared to mint, oregano, parsley, rosemary, sage, bean stew, onions or garlic. Its cell reinforcement adequacy was much more than that of BHA (butylhydroxyanisol) or BHT (butylhydroxytoluene). There are numerous compounds in charge of the cell reinforcement impact of basil, for example, â-carotene, tocopherol, eugenol, isoeugenol, linalool and linalyl acetic acid derivation and additionally flavonoids [5].

Thyme (*Thymus vulgaris*), belongs to Family *Lamiaceae*, is a fragrant and has therapeutic role [8]. Known essential components of the thyme incorporate key oil, tannin, flavonoids, saponins, and triterpenic acid. Rosemary (*Rosmarinus officinalis*) is used to cure several diseases such as asthma, chronic indigestion, colon toxins, fatness, sinus, congestion, fever, cold

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extremities, colic, gastric disorders and diarrhea. Basil (*Ocimum basilicum*) is found locally in Asia and is used as a valued flavor enhancer since antiquated eras. *Ocimum basilicum* is reported to have antimicrobial property. Both aqueous and ethanolic concentrates of basil have reported for antibacterial activity against penicillin resistant strain of some microorganisms [9]. Keeping this in view, the determination of bioactivity potential of extracts of *Ocimum basilicum*, *Thymus vulgaris, Rosmarinus officinalis* and *Origanum vulgare* was evaluated against ampicillin resistant Gram negative bacteria. These plants were selected due to their widespread use as spices and easy availability throughout the world. The aim of the study is to search a potential source for new type of bioactive compounds which can be used to cure diseases caused by antibiotic resistant bacteria.

MATERIALS AND METHODS

Collection of Plant Material

Fresh mature leaves of *Ocimum basilicum, Thymus vulgaris, Rosmarinus officinalis* and *Origanum vulgare* were separately collected from local nursery of Garden town, Lahore (31° 30' 34" North, 74° 19' 22" East, Punjab, Pakistan, Asia). The plants were identified taxonomical and authenticated. The leaves from the plants were taken in a large quantity, washed and dried in an oven at 60ºC for five minutes for the extraction purposes.

Preparation of Plant Extracts

The dried leaves were mechanically ground separately and then crushed with the help of electrical crusher into powder form. Each plant material was mixed inethanol (Merck) and water separately at a ratio of 1:4 (w/v) and kept on shaker for 72 hours in properly labeled and sealed conical flasks. The extracts were filtered. The ethanol filtrate was then evaporated using a rotary evaporator (Hei-vap-series, Heidolph Germany). These dried extracts were re-suspended in ethanol. Aqueous and ethanolic extracts having concentrations of 100 mg/ml were stored in refrigerator until further analysis.

Collection of Fruits and Vegetables

Four different fruits and vegetables were collected from local markets and supermarkets. The collected fruits were apples, peaches, grapes, persimmons (japani pal) and vegetables were lettuce, carrots, tomatoes, and peppers. Skin of these fruits and vegetables were peeled and placed in L-broth and incubated for 24 hours at 37^{o}C.

Isolation, Characterization and Ribotyping of Ampicillin Resistant Bacterial Strains

For the isolation of ampicillin resistant bacteria from these fruits and vegetables, serial dilution method was used. For this purpose, the samples were serially diluted and 50µl of each dilution was spread plated on ampicillin (150 and 250µg/ml) supplemented L-agar plates. The plates were then incubated at 37°C for 2-3 days. Morphologically different bacterial strains were characterized morphologically and biochemically [10]. Normally, these strains were maintained on Luria Bertani (LB) agar (pH 7.0) at 37 °C. For ribotyping, bacterial strains were sent to Macrogen (Seoul, Korea). Sequences of the bacterial strains were analyzed using the Ribosomal Database Project. Neighbor-joining method was used to interfere with the evolutionary history [11]. An evolutionary analysis was conducted in MEGA5 [12].

Phytochemical Screening of Plant Extracts

Alkaloids

To check the presence of alkaloids, plant sample extract (0.5g) was agitated with 1% HCl on a water bath. The obtained solution was filtered. Two drop of Mayer's reagent were added to 1ml of the filtrate and made the final volume up to 100 ml with distilled water. Turbidity of the extract filtrate on addition of Mayer's reagent confirmed the presence of alkaloids.

Saponins

For the presence of saponins in plant extracts, one ml of each extract was mixed with nine ml of distilled and shaken vigorously for 15 seconds. Let it stands for 10 minutes at room temperature. Presence of saponins was confirmed by formation of stable foam.

Flavonoids

To test the presence of flavonoids in plant extracts, 0.5 ml of the extract and a small number of drops of $MgCl_2$ were mixed with concentrated HCl. Presence of flavonoids was indicated by the development of a faint orange color.

Tannins

To detect the tannins in plant extracts, 0.5 ml of the plant extract and 1 ml of distilled water was stirred and filtered. Ferric chloride solution (5 %) was added to the

filtrate. Appearance of blue black or blue green precipitate indicated the presence of tannins.

Coumarin

For the determination of coumarin, 10% of NaOH and chloroform were added to the test sample. Development of yellow color indicated the existence of Coumarin.

Quinone

For the detection of quinone, sodium hydroxide was added to the test substance. Blue green or red color showed the presence of quinone.

Phlobatannins

The extract (0.5 g) was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCl solution. Formation of red precipitate confirmed the presence of phlobatannins.

Thin-Layer Chromatography (TLC)

Thin layer chromatography (silica gel coated, Merck) was performed for the characterization of ethanolic plant extracts. For the detection of flavonoids solvent system used was chloroform: acetic acid: methanol: water in ratio of 6.4: 3.2: 1.2: 0.8. Whereas solvent system consisted of chloroform: ethyl acetate: methanol in ratio of 6: 4: 0.3 was used to confirm the presence of saponins. These components were visualized under visible and UV light (254 nm and 366 nm) and spots were marked. Plates were sprayed with $SbCl_3$/chloroform spray and $CuSO_4$ solution spray. Silica gel was chosen as a stationary phase since it was efficient absorbent for TLC separation of most of the plant extract.

Screening for Antibacterial Activity

Agar well diffusion method was used for the determination of antimicrobial activity of ethanolic and aqueous extracts [13]. For this purpose, wells of five mm diameter were made on sterile Muller-Hinton agar (MH) plates by using sterile Pasteur pipette. These plates were equally swabbed with overnight culture of isolated ampicillin resistant strains. About 100 µl of each of the extract (ethanolic and aqueous extract) was dispensed into each well and then incubated at ± 37°C for 24 hours. Antibacterial activity was determined in terms of zones of inhibition (mm). Ethanol or autoclaved distilled water (100 µl) was used as negative control. The antagonistic effect of each extract was tested against ampicillin resistant strains in replicates.

Anti-Mitotic Activity

Antimitotic activity was assessed by using *Allium cepa* roots meristematic cells. Small onion bulbs were grown in tap water for 48 hours at room temperature. The bulbs with uniform root development were selected for the experiment. The roots were treated with the prepared extracts. Distilled water was used as control. The different treatments used were given in table. After 24 hours of treatment, the root tips were cut and transferred to the fixative solution of glacial acetic acid and absolute alcohol (1/3 v/v). Squash preparations were done by staining the treated roots with gimsa stain. MI (Mitotic index) was calculated.

Statistical Analysis

The data obtained was statistically analyzed using SPSS personal computer statistical package (version 16, SPSS Inc, Chicago).

RESULTS

Isolation of Antibiotic Resistant Bacterial Strains

Ampicillin resistant bacteria were isolated from fruits (apple, peach, grapes and persimmon) and from vegetables (lettuce, carrots, tomatoes and peppers) on ampicillin (150µg/ml and 250µg/ml) supplemented L-agar plates by serial dilution method. Total three morphologically different strains (A, P and L) were selected for further study on the basis of their high ampicillin (250µg/ml) resistance.

Characterization and 16S rRNA Analysis of Bacterial Isolates

Isolated bacterial strains were subjected to morphological and biochemical characterization. These strains were found to be gram negative rods, motile, non-spore and non-capsule formers. They presented positive results for catalase, oxidase, whereas they were negative for MR-VP (Methyl Red, Voges-Proskauer), indole production and urease tests. Isolated bacterial strains (A, P and L) exhibited resistance for all antibiotics used. In order to make comparison between the 16S rRNA gene sequences with those in the National center for Biotechnology Information (NCBI) sequence database (GenBank), BLAST (http://www.ncbi.nlm.nih.gov/BLAST) was used. The 16S rRNA sequences of the strains A (KR080312),

Table 1: Phytochemical Screening of Crude Extracts of Selected Medicinal Herbs

Plant Extract	Bioactive Compounds						
	Alkaloids	Saponins	Coumarin	Phlobotannins	Quinones	Flavonoids	Tannins
Basil	+	+	+	+	+	+	+
Thyme	+	+	-	+	-	+	+
Rosemary	+	+	-	+	+	+	+
Oregano	+	+	-	+	-	+	+

Key: (+) Present, (-) Absent.

P (KR080311) and L (KR095628) showed similarity to that of *Pseudomonas aeruginosa* strain A, *Pseudomonas aeruginosa* strain P and *Rhodobacter sphaeroides*, respectively.

Phytochemical Screening of Plant Extracts

Numerous secondary metabolites or phytochemicals are produced by plants to secure themselves from predation by microbes, parasites and herbivores. Phytochemical screening of selected plant samples is presented in Table **1**. Alkaloids, saponins, phlobotannins, flavonoids and tannins were found in all extracts. Coumarin were found in basil only whereas quinones were present in basil and rosemary.

Screening for Antibacterial Activity

In order to check the antibacterial activity of extracts of *Ocimum basilicum* (basil)*, Thymus vulgaris* (thyme)*, Rosmarinus officinalis* (rosemary) and *Origanum vulgare* (oregano) against ampicillin resistant isolates, agar well diffusion method was used. Inhibitory effect was observed in terms of diameter of zones of inhibition (mm). Ethanol was used as control. The results displayed that the crude ethanolic extracts inhibited the bacterial growth with remarkable zone of inhibition (15 mm) as compared to the aqueous extracts (10 mm). The order of inhibitory effect of crude extracts was as follows:

Ethanolic Extracts > Aqueous Extracts

In all types of extracts (ethanolic and aqueous), Thyme (*Thymus vulgaris*) showed maximum diameter of zone of inhibition (15 mm) in comparison with rest of the studied medicinal plants. This showed that thyme plant extract was more effective in inhibiting the growth of ampicillin resistant bacterial strains (A, P and L) thus had good antibacterial activity. The ethanolic extract of basil and oregano showed largest zone of inhibition (10 mm). The smallest zone of inhibition of (5 mm) was exhibited by rosemary. Ethanolic and aqueous extracts of Thyme showed largest zone of inhibition against Strain Las compared to P and L strains. The ethanolic extracts showed zones of inhibition in the range of 6-15 mm against strain A, P and L. While aqueous extracts

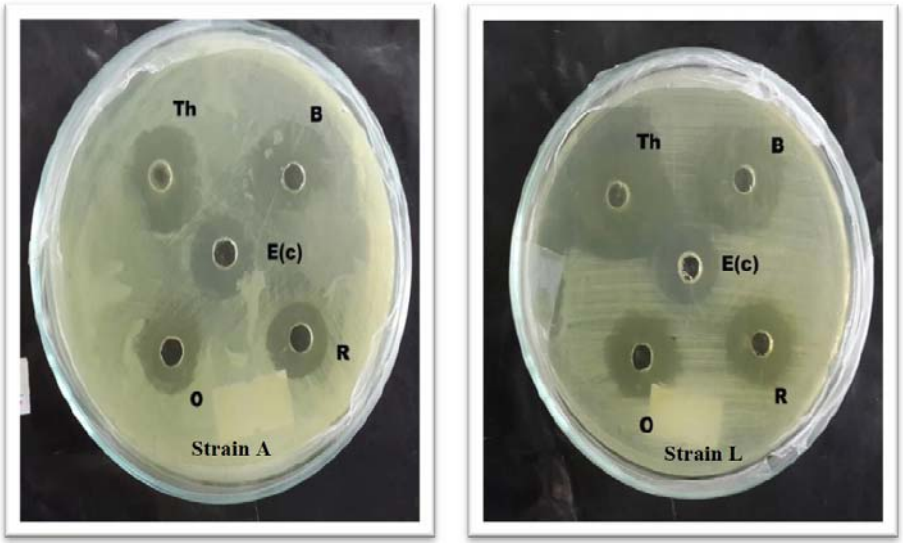

Figure 1: Antibacterial activity of crude ethanol extracts of medicinal plants against ampicillin resistant bacterial strains A and L by Well Diffusion method.

Table 2: Antibacterial Activity of Crude Ethanolic and Aqueous Extracts of Medicinal Herbs Against Ampicillin Resistant Bacterial Isolates

Strains	Solvent	Zone of Inhibition (mm)			
		Basil	***Thyme***	***Rosemary***	***Oregano***
A	Ethanol	6 ± 6.50	11 ± 0.71	10 ± 0.55	9 ± 1.26
	Water	5 ±1.06	7 ± 0.45	6 ± 1.24	6 ± 0.56
P	Ethanol	10 ± 0.44	12 ± 2.14	9 ± 0.12	8 ± 0.75
	Water	5 ± 0.28	9 ± 0.90	7 ± 1.24	5 ± 4.41
L	Ethanol	10 ± 0.71	15 ± 1.85	12 ± 0.75	10 ± 0.2
	Water	7 ± 1.41	10 ± 0.71	8 ± 4.41	9 ± 1.26

Mean of three triplicates, ±standard error of the mean.

showed zones of inhibition in range of 5-10 mm in all strains, with the largest zone of inhibition against the strain Las compared to rest of the ampicillin resistant isolates (Figure **1**, Table **2**).

Thin-Layer Chromatography (TLC)

TLC divided up components of analyst on the basis of different travelling rates of numerous components due to the differences in their attraction to the stationary phase, and because of differences in solubility in the mobile phase. The results showed a large number of bands when plate was observed under high and low UV, respectively. When the plate was sprayed with $CuSO_4$, Na_2CO_3 and tri-sodium citrate solution, the reddish orange bands were observed that confirmed the presence of saponins in ethanolic extracts of these medicinal plant leaves. The appearance of greenish-black bands, when the plate was sprayed with $SbCl_3$ solution showed that ethanolic extracts of these medicinal plant leaves contained flavonoids (Figure **2**).

Anti-Mitotic Activity

Antimitotic agents can cease the mitosis in any phase of the cell cycle. Aqueous extract (100%) of thyme reduced mitotic index significantly after 1 day extract treatment (10 mg/ml) in comparison with rest of the extracts. As the mitotic index showed gradual decrease with rise in the concentration of the extracts. Control roots exhibited normal process of mitosis. Therefore, the aqueous extracts showed comparatively lower mitotic index than the control. 100% aqueous extract exhibited significant antimitotic activity by reducing the mitotic index ($P<0.01$) from 80 % to 23.1 % after treatment of a day at a concentration of 10 mg/ ml. Mitotic index of thyme was found to be 23.11 %, basil 39.33 %, rosemary 40% and oregano 45 % after a day treatment, respectively (Figure **3**). So, aqueous extract of Thyme was found to be most effective against cells that were multiplying rapidly and created cytotoxic effect either by blocking the formation of the mitotic spindle in M-phase or by damaging the DNA during the S-phase of the cell cycle.

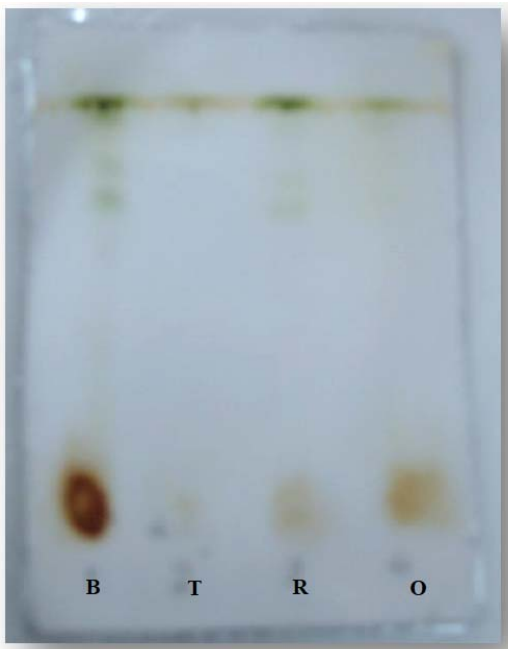

Figure 2: TLC plate with bands showing presence of flavonoids in these medicinal plants leave extracts. (B = Basil; T = Thyme; R = Rosemary; O = Oregano).

DISCUSSION

The present study was intended at determination of antibacterial potential of *Ocimum basilicum, Thymus vulgaris, Rosmarinus officinalis* and *Origanum vulgare* on growth of ampicillin resistant isolates. Phytochemical analysis was done in order to confirm

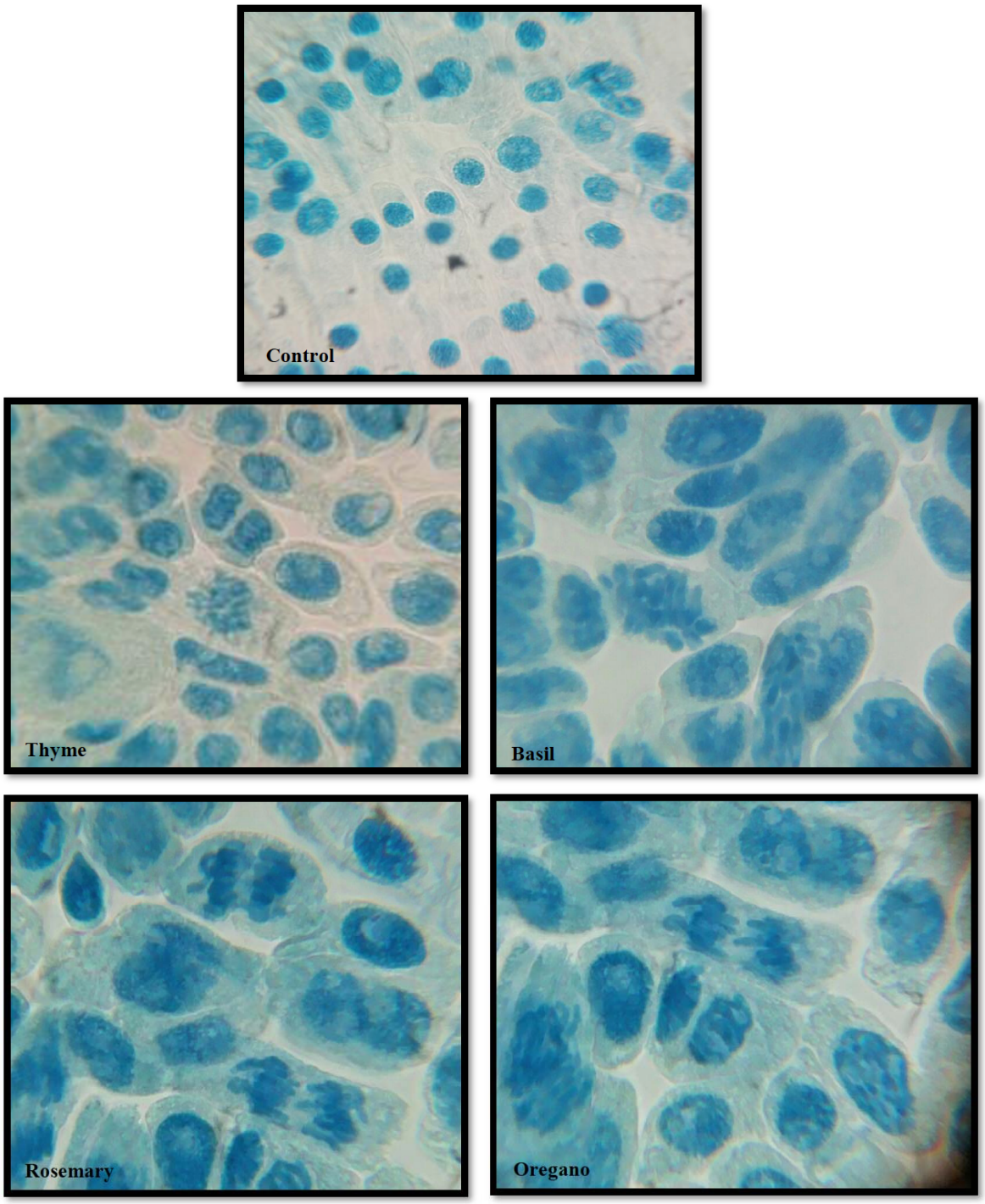

Figure 3: Extracts of herbs exhibited significant antimitotic activity on roots of *Allium cepa*, in compassion with control.

the presence of alkaloids, flavonoids, tannins, phlabotanins, quinones, coumarin and saponins in the extracts of basil, thyme, rosemary and oregano. The results showed that crude extracts of basil contained all the studied bioactive compounds. While the extracts of thyme and oregano had alkaloids, flavonoids, tannins, phlabotanins and saponins. Rosemary contained all the bioactive compounds expect for coumarin. Moreover, in the previous studies it was also reported that mostly plant extracts contain alkaloids, flavonoids, terpenoids, phlabotanins, tannins, saponins and reducing sugars [14].

TLC showed that extracts of *Ocimum basilicum, Thymus vulgaris, Rosmarinus officinalis* and *Origanum vulgare* contains many compounds as many different bands were observed under low and high UV light. Only the saponins and flavonoids were detected when

plate was sprayed with $CuSO_4$ solution as well as with $SbCl_3$ solution. The saponins as well as flavonoids are present in different plant parts and protect plant from predators as well as parasites. The previous studies on antimicrobial potential of plant secondary metabolites supported the role of saponins and flavonoids as defensive compounds [15]. Antimicrobial activity of herbal plants is greatly influenced by the kind of solvent selected for the extraction. In general, extraction of herbs with ethanol leads to better antimicrobial activity than extraction with water. This is might be due to the fact many of the antimicrobial compounds are aromatic or saturated organic compounds in nature and are highly soluble in polar solvents such as ethanol [16]. The antibacterial potential revealed that all extracts were potent antimicrobial against all ampicillin resistant strains studied. Ethanolic extracts showed a high degree of inhibition than aqueous extract. The ethanolic extracts revealed highest antimicrobial activity indicating the polar nature of bioactive compounds, possibly polyphenols or aldehydes which caused the inhibition of these ampicillin resistant strains [17]. The observed antimicrobial activity of these herbal extracts against studied ampicillin resistant strains might be due to the occurrence of tannins and flavonoids as these have been previously reported to possess antimicrobial activities. Whereas, the slower action of other extracts as antimicrobial agents may be caused by the slight diffusion of certain extracts in the agar [5].

Previously, Cimanga *et al.* [18] reported the antimicrobial activities of alkaloids against numerous microorganisms [19]. Largest zone of inhibition was given by crude ethanolic extracts of thyme against *Rhodobacter sphaeroides* (L strain). While smallest zone of inhibition was by crude aqueous extracts of basil and oregano against A and P ampicillin resistant strains. The low antimicrobial action of aqueous extracts could be attributed to low level of anionic components such as nitrate chloride, thiocynate and sulphates along with other water soluble components found certainly in majority of plant materials [5]. The highest inhibitory activity of ethanolic extracts, suggested that the active component of medicinal plants (*Ocimum basilicum, Thymus vulgaris, Rosmarinus officinalis* and *Origanum vulgare)* may be a highly polar compound. Previously, there are reports that describe the ethanol to be the best solvent for the extraction of most medically important antimicrobial components [16, 20-22].

In general, data revealed that among the three tested ampicillin resistant organisms, *Pseudomonas aeruginosa* (strain A and P) and *Rhodobacter sphaeroides* (Strain L) were more susceptible microorganism to the extracts from *Ocimum basilicum, Thymus vulgaris, Rosmarinus officinalis* and *Origanum vulgare.* However, the exact mechanisms by which microbes endure the action of antimicrobial agents are not well understood and remained dubious [23].

Mitosis is a method of division of somatic cells. Mitosis is aimed for the duplication of cell number in embryogenesis and blastogenesis of plant and animals. Anti-mitotic agent is any agent that stops or disturbs mitosis, so that is helpful in treating life-threatening diseases like cancer [24]. The antimitotic activity of all four extracts showed potential for inhibiting the growth of cells (Figure **4**). Inhibition of mitosis results due to binding of the extract with the cell

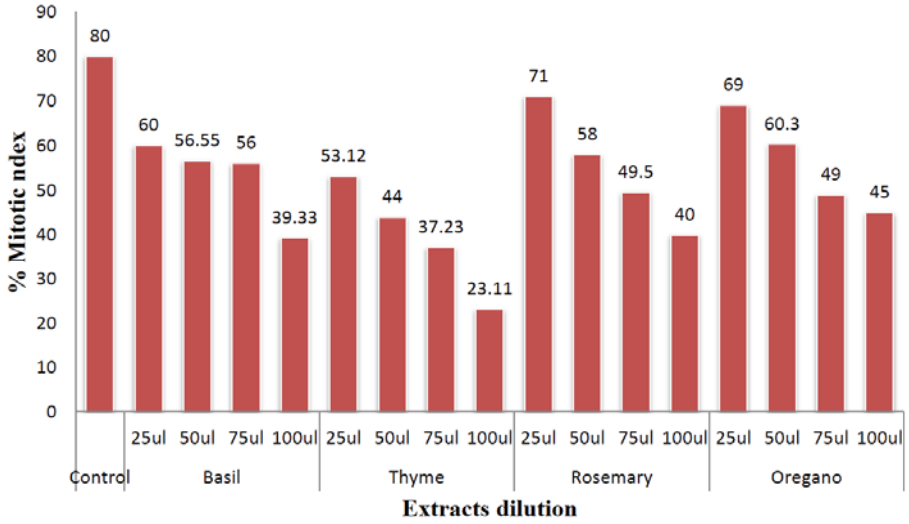

Figure 4:

proteins that will bring out cell division [25] and this may be due to existence of glycoside particularly anthraquinone glycosides, and phenolic compounds accountable for anti-mitotic activity [24]. Several anticancer drugs of plant origin are identified to be effective against rapidly multiplying cancer cells. They affect the cell kinetics and hence exhibit the cytotoxic effect. However, due to side effects of most of the cytotoxic drugs need of the hour is to develop drugs that are effective with fewer side effects such as herbal medicine [26].

CONCLUSION

These findings revealed the antibacterial potential of all tested extracts against ampicillin resistant bacterial strains. Ethanolic as well as aqueous extract of *Thymus vulgaris* was found best with maximum bacterial inhibitory activity. Moreover, anti-mitotic activity of these extracts is helpful for inhibition of the growth of cancer cells. This scientific information requires clinical trials of these bioactive compounds to benefit the people of developing countries such as Pakistan.

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